PHYSICAL AND CHEMICAL CHARACTERISTICS OF CHLOROPLAST FRAGMENTS

Applied Research Concerning Artificial Photosynthesis

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FOREWORD

This program was conducted at Armour Research Foundation of Illinois Institute of Technology, Technology Center, Chicago 16, Illinois, for the 6570th Aerospace Medical Research Laboratories, under Contract AF 33(616)-7255. The program was initiated and monitored by Dr. A. R. Slonim and Dr. K. Yokoyama of the Life Support Systems Laboratory, 6570th Aerospace Medical Research Laboratories, under Project No. 6373, "Equipment for Life Support in Aerospace," Task No. 637301, "Applied Ecology." It was subsequently monitored by 1st Lt. Paul A. Lachance and Dr. Alton E. Prince of the Biospecialties Section, Physiology Branch, Biomedical Laboratory, 6570th Aerospace Medical Research Laboratories, in support of Project No. 7164, "Space Biology Research," Task No. 716403, "Environmental Biology." At Armour Research Foundation the research program was planned and conducted by Dr. J. A. Gross, Mr. M. J. Becker, and Mr. A. M. Shefner. This has been designated Report No. ARF 3174-24. The studies were conducted from 1 November 1961 to 14 September 1962. The authors greatly appreciate the advice and assistance of: Dr. W. L. Butler, U. S. Department of Agriculture, Beltsville, Maryland, for studies by low temperature spectrophotometry; Dr. Irene Corvin, Solid State Physics Section, Armour Research Foundation, for x-ray analyses and Mrs. B. Tooper and Mr. C. Nagamoto, respectively, of Solid State Physics and Fine Particles Section, Armour Research Foundation for electron microscopy.

ABSTRACT

Chloroplasts isolated from spinach leaves were physically fragmented and then fractionated by differential centrifugation. A high-activity fraction, designated CF_{20-50}, was obtained and retained its high activity regardless of environmental changes. It had a low protein content and a relatively high chlorophyll-to-protein ratio. Plastoquinone was present in high concentration. Extraction of plastoquinone from CF_{20-50} inhibited Hill activity. Unlike whole chloroplasts, readdition of plastoquinone failed to induce even partial recovery.

The average particle size in CF_{20-50} was 1500 A diameter. The average sedimentation constant was 800; the molecular weight was about 8×10^7; and it showed three electrophoretic bands.

CF_{20-50} showed maximal Hill activity, but was not the smallest photoactive unit studied. Although the nature of the smallest functional unit is uncertain, it is clear that a CF_{20-50} particle is composed of smaller sub-units probably oriented to impart high activity to the larger composite structure.

PUBLICATION REVIEW

This technical documentary report has been reviewed and is approved.

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APPLIED RESEARCH CONCERNING ARTIFICIAL PHOTOSYNTHESIS

I. INTRODUCTION

The research effort of this project was designed to develop fundamental information about the oxygen-evolving mechanism of the photosynthetic structures of plants, the chloroplasts. The approach to this problem, as outlined in the MRL-TDR-62-5 (ref. 18), was to correlate the chemistry and the structure of chloroplast fragments with their functional capacities. Investigations employing this approach were continued through the present contract year and are the subject matter of this report.

In the initial research period it was shown that a particulate fraction, differentially centrifuged from isolated ruptured spinach chloroplasts, possessed a high Hill reaction rate, which is defined as the rate of oxygen evolution and/or the rate of transfer of electrons to an added acceptor upon illumination. This fraction, which could not be sedimented from dilute saline by centrifugation at 20,000 x g for 10 min but was sedimented by centrifugation at 50,000 x g for 10 min, was designated CF_{20-50}. Comparative studies with other fractions which were sedimented at lower or higher g forces revealed that CF_{20-50} had not only the highest level of Hill activity but also had a high chlorophyll-to-protein ratio. Therefore, there was a positive correlation between activity and the ratio of pigment to protein.

The average size of a CF_{20-50} fragment was estimated to be a 1500-A-diameter particle, which could accommodate about 16,000 chlorophyll molecules on its surface. This size is extremely large in comparison with the smallest units which are theoretically capable of photosynthetic function.

To increase the basic information about these highly active oxygen-evolving units, investigations were centered on those physical and chemical characteristics which might be related to the activity differences between fractions. Thus, factors correlating with maximal activity might be identified. Quantitative measurements were made of the chlorophyll, protein, and plastoquinone content of the chloroplast fragments. The lipid content was estimated. Particle sizes were measured from electron micrographs. Ultracentrifugal analyses were performed. A little-explored method of electrophoresis was used. Studies by x-ray diffraction were attempted.

This research yielded new information about the oxygen-evolving substructure of the chloroplast. Continued investigation in this laboratory coupled with information emanating from other research groups studying photosynthesis can increase the probability of the successful development of an artificial photosynthetic system.

II. PREPARATION OF MATERIALS

With a few modifications, the preparatory techniques are essentially as reported previously (refs. 2, 18). Briefly, fresh spinach was bought at a local market and washed in cold water. Leaves were cut from the stem, deribbed, and minced. The minced leaves were ground in a Waring blender in a sucrose-phosphate buffer, and filtered through cheesecloth. From this homogenate, intact whole chloroplasts (WC) were isolated by centrifugation and were osmotically shocked by suspending them in 0.015 N unbuffered sodium chloride. The chloroplast suspension was disintegrated further in a Raytheon magnetostrictor. The sonicated suspension of chloroplast fragments, CF_{OS}, served as the starting material from which particulate fractions were separated by differential centrifugation.

The fractions of chloroplast fragments (CF) are designated by subscripts which denote the centrifugal forces (g x 10^3) which were used for isolation. Thus CF_{20-50} refers to a fraction sedimented by centrifuging the supernatant of a 20,000 x g spin at 50,000 x g.

The major points of difference between the present and prior methods (refs. 2, 18) of preparation are:

(1) Washing in sucrose buffer and recentrifuging the isolated chloroplasts at 200 x g for 2 min

(2) Three 3-min sonication periods instead of two

(3) Washing and repelleting of each experimental fraction.

III. ASSAY METHODS

A. Chemistry

1. Chlorophyll

a. Total

Chlorophyll was determined by the method of Arnon (ref. 1) as described previously (ref. 18).

b. Types of Chlorophylls and Their Ratios

As an indicator of chlorophylls and other pigments, the ratios of extinction values at absorption peaks were calculated from absorption spectra scanned with the Beckman DK-1 or Cary model 14 recording spectrophotometers.

Low-temperature absorption and fluorescence spectrophotometry of chloroplast fractions were obtained in collaboration with Dr. W. L. Butler.* This investigation distinguished peaks of chlorophylls a and b and the long-wavelength chlorophyll C-705 (ref. 6).

2. Protein

Total nitrogen was measured and protein was calculated according to the method described previously (ref. 18).

3. Dry-Weight Measurements

Fractions were either dialyzed against distilled water or centrifuged and washed with distilled water to remove soluble interfering compounds prior to dry-weight analyses. Chlorophyll and protein were analyzed before and after dialysis to ascertain any losses resulting from the dialysis procedure. Recoveries for both components were between 90 and 95% after a 4-hr dialysis, thus giving adequate assurance that no undue errors were being introduced. Subsequent to dialysis, the nondialyzable material was carefully rinsed from the sac into a tared aluminum weighing dish and dried at 90 to 100 °C under vacuum for several hours. The samples were then dried to constant weight at atmospheric pressure in an oven at 110 °C. After removal from the oven, they were permitted to cool to room temperature in a desiccator and were weighed on a standard analytical balance to the nearest 0.1 mg.

As a check on the validity of the results obtained from the above method, a second technique was devised. A portion of each chloroplast fraction was centrifuged for 1 to 2 hr at 145,000 x g in the Spinco model L preparative ultracentrifuge, the time of centrifugation being dependent on the fraction; e.g., CF_{20-50} was spun for a shorter time than CF_{70-145}. The resultant pellet was

*Personal communication from Dr. Warren L. Butler, Instrumentation Research Laboratory, Agricultural Marketing Service, U.S. Department of Agriculture, Beltsville, Maryland.

rinsed with distilled water, so that most of the residual solids from the original suspending medium were eliminated. The washed pellet was suspended in 5 to 10 ml of distilled water, rinsed into a weighing dish, dried, and weighed as described above.

The agreement between the two methods of dry-weight determination was excellent.

4. Plastoquinone

a. Extraction

Various methods have been employed by other investigators for extraction of plastoquinone (PQ) (refs. 4,5,7,8,25,31). Consequently, our previous method (ref. 18) was changed to take advantage of the newer information gleaned from the published literature and from discussions with Dr. F. L. Crane.* The procedure adopted was a modification of that used by Kegel and Crane for extraction of another quinone, vitamin K, (ref. 23), and has been described briefly by Becker et al. (ref. 3).

Unless otherwise noted, all procedures for the extraction, isolation, and assay of PQ were carried out at room temperature under a dim green fluorescent light which has a single sharp energy peak at 5461 A and a bandwidth from 4900 to 5850 A encompassing 90% of its available emitted energy. Aqueous suspensions of chloroplast fractions (2.6 volumes) containing 1.5 to 3.0 mg of chlorophyll in phosphate buffer at pH 6.8 were shaken for 3 hr with 1 volume of n-heptane-isopropanol (1:1, v/v). The suspension was centrifuged for 10 min at 2000 rpm, and the deep green upper organic phase was withdrawn and transferred to another flask. The lower aqueous phase and residue were rinsed into the original extraction flask with 2 volumes of n-heptane and agitated for another 1/2 hr. The suspension was again centrifuged, and the green upper phase was pooled with the first. The procedure was repeated twice more until the final organic phase was pale green and the particulate residue was pale green to beige-colored, depending on the starting material.

The extract, containing chlorophyll, PQ, and other fat-soluble components, was concentrated to dryness by flash evaporation at 40 to 45 °C. The dried extract was stored at -20 °C under nitrogen until processed further.

b. Isolation

The dry extract was dissolved in 5 to 8 ml of n-heptane and placed on a 1-cm-diameter column of Decalso (50 to 80 mesh) which had been prewashed with 150 to 200 ml of n-heptane just prior to use. The amount of Decalso in the column was calculated according to the method of Crane (ref. 7) and was 4 g per mg of chlorophyll in the starting material. The column, with a 10 to 15-ml holdup volume, was all-glass with Teflon fittings to avoid contact between the organic solvents and rubber, soluble plastic, or stopcock grease which might interfere with isolation and subsequent analyses.

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*Personal discussions with Dr. Frederick L. Crane, Department of Biological Sciences, Purdue University, W. Lafayette, Ind.

The extract was allowed to percolate through the column until no more than 0.5 ml remained above the adsorbent surface. The column was washed with 35 to 45 ml of n-heptane, and about 45 ml of eluate containing no PQ was discarded. Then 40 ml of a mixture of n-heptane and ethyl ether (9:1, v/v) was added. The first 5 to 10 ml of eluate was discarded. The following eluate of 20 to 25 ml, containing essentially all the elutable PQ, was saved as the "PQ fraction." Subsequent washes of the column with mixtures of n-heptane and ethyl ether in ratios of 9:1, 8.5:1.5, and 8:2 did not elute any more PQ. The PQ fraction was dried by flash evaporation and stored under nitrogen at -20 °C.

c. Assay

The dried PQ fraction was dissolved in 2 to 4 ml of absolute ethanol. This ethanol solution, which had a yellow-orange to pale yellow color, showed an absorption maximum near 255 mμ, indicating that PQ in the oxidized form had been eluted from the Decalso column. When necessary, because of high concentration, the ethanolic PQ solution was diluted with 100% ethanol to bring the optical density (E) between 0.2 and 0.6 at 255 mμ. A few crystals of potassium borohydride were then added directly to the PQ fraction in a cuvette in order to obtain the spectral curve for reduced PQ. The reference solutions were absolute ethanol with or without potassium borohydride. The oxidized and reduced spectral scans for the PQ fraction were obtained in a Beckman DK-1 recording spectrophotometer. Figure 1 shows a representative curve of the PQ fraction from whole chloroplasts. Calculations for the concentration of PQ were made by substituting the appropriate E_{255} and E_{275} values from the curve into the equation developed by Crane (ref. 8). After the PQ content of a given ethanolic fraction was determined, the remaining portion of the fraction was pooled with other PQ fractions and stored under nitrogen at -20 °C for reconstitution experiments.

d. Reconstitution with PQ

The procedure used for reconstitution of PQ-extracted materials is outlined in a flow diagram (fig. 2). All steps in the procedure were carried out on fresh chloroplast fractions under dim green light. The temperature was kept below 5 °C.

Whole chloroplasts or a chloroplast fragment suspension containing between 2.5 and 10.0 mg of chlorophyll was shell-frozen and lyophilized to dryness. To the lyophilized material 5 ml of petroleum ether was added per 2.5 mg of chlorophyll. The mixture was mechanically shaken for 5 to 10 min and then centrifuged in an International PR-2 centrifuge for 5 min at 2000 rpm. The supernatant was discarded. The residue was partially dried under a gentle stream of dry, oil-pumped nitrogen; drying was completed by lyophilization. The resultant powder was suspended in 3 to 5 ml of petroleum ether and divided into two aliquots. Enough of the pooled PQ fraction, dissolved in petroleum ether, was added to one aliquot to bring the PQ content to 0.1 mole per mole of chlorophyll. An equivalent volume of pure petroleum ether was added to

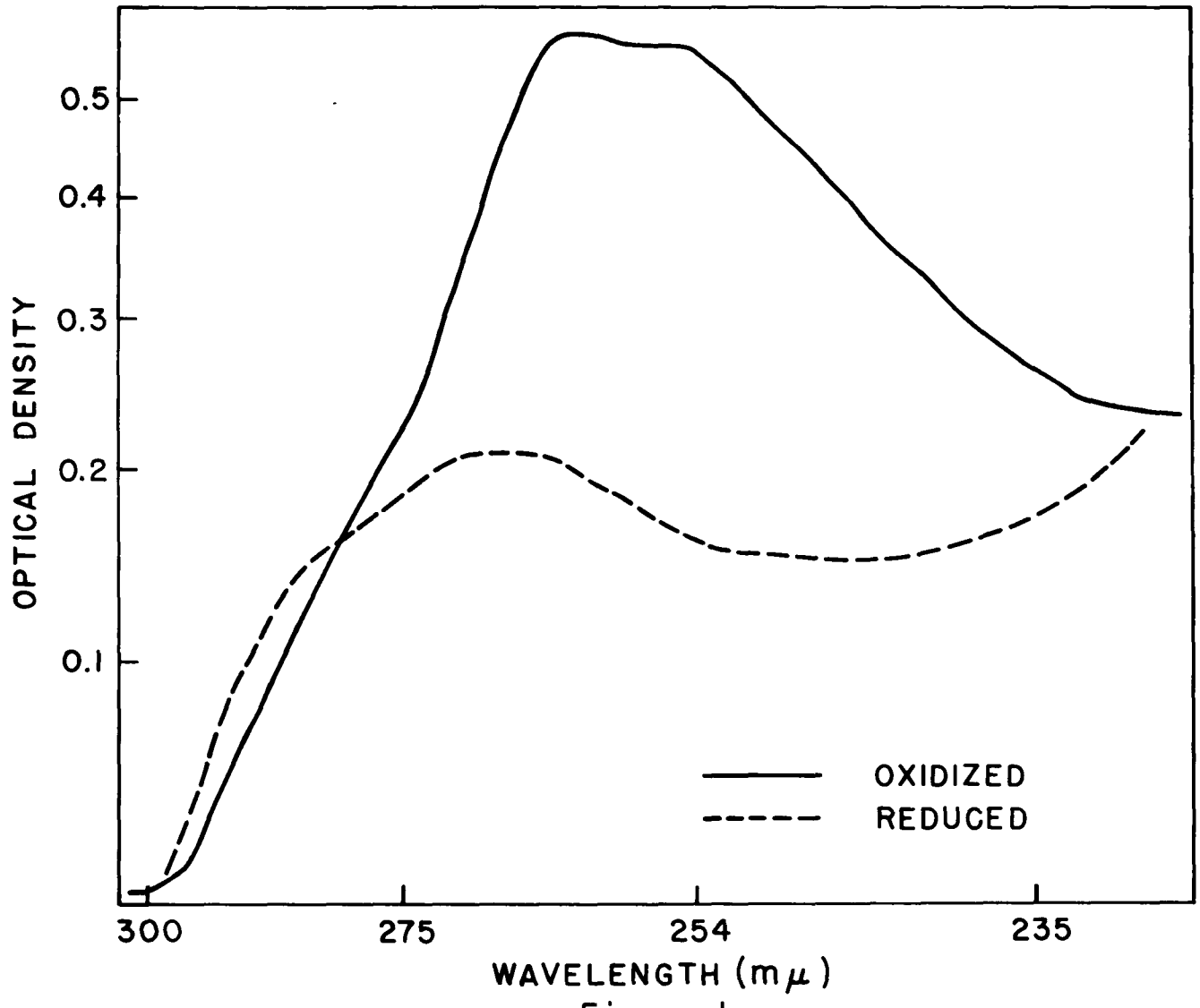
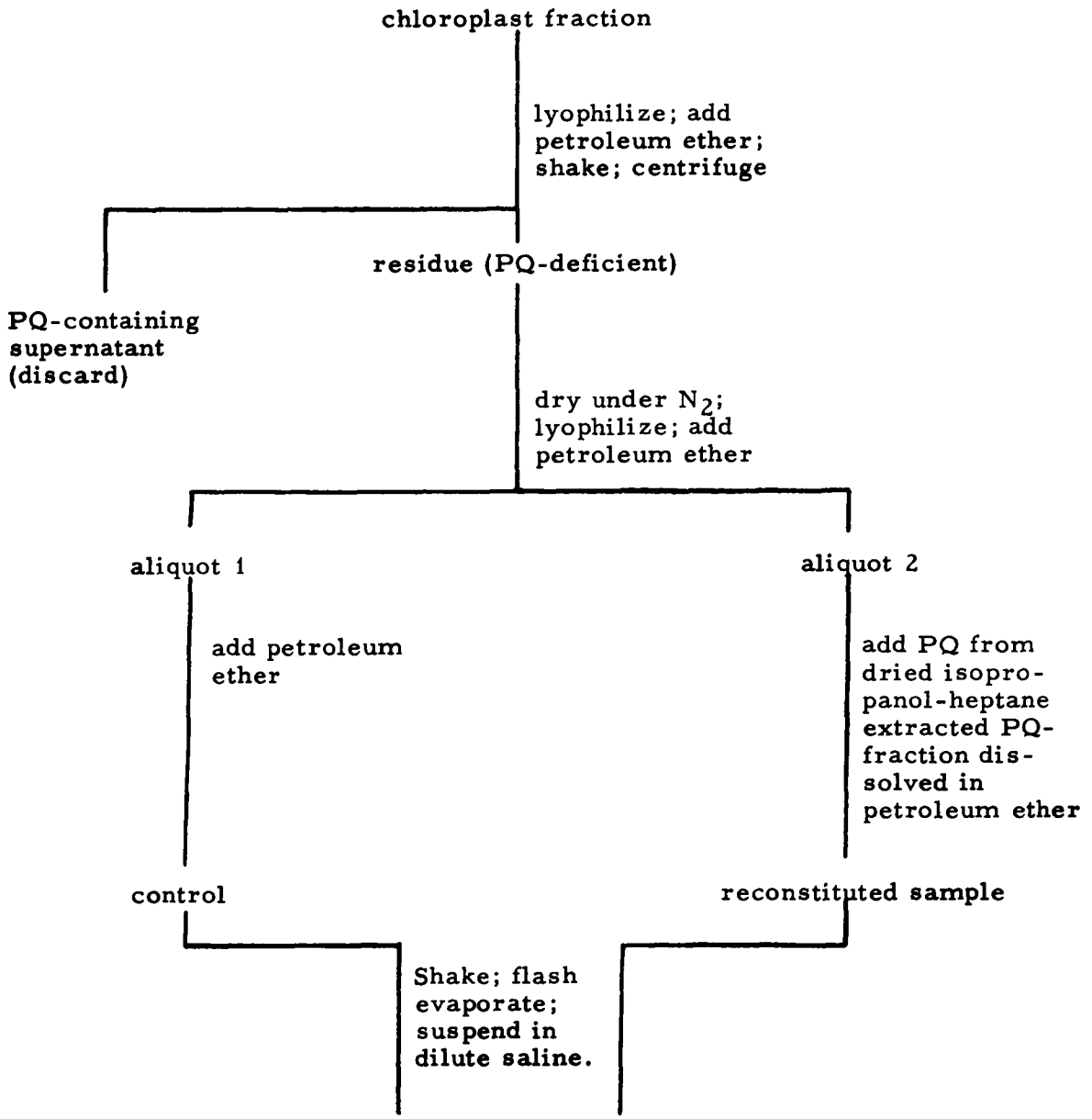


Figure I

ABSORPTION SPECTRA IN ETHANOL OF PLASTOQUINONE EXTRACTED FROM WHOLE CHLOROPLASTS



1. Assay for chlorophyll
2. Assay for Hill activity

Figure 2

RECONSTITUTION OF CHLOROPLAST FRACTIONS WITH PLASTOQUINONE

the control aliquot. These reconstituted mixtures were shaken for 15 to 20 min, flash-evaporated to complete dryness, and suspended in aqueous solution for assay of Hill activity. The chlorophyll content in each aliquot was again determined and activity was based on this value rather than the initial value, since some chlorophyll was lost with the original petroleum ether supernatant.

5. Cytochromes

Three methods were used to determine the presence of cytochrome in chloroplast fragments.

The first technique was slightly altered from the one suggested by Davenport and Hill (ref. 10) for the extraction of cytochrome f from leaves. The chloroplast fragments, suspended in 0.015 N sodium chloride, were extracted with an equal volume of 97% ethanol containing 1.5% concentrated ammonium hydroxide solution (sp. gr. 0.90). Acetone (1.1 volume per volume of ethanol extract) was added to precipitate the cytochrome, and the mixture was filtered through kieselguhr. The precipitate adsorbed to the kieselguhr was washed with a 2:1:1 mixture of acetone, ethanol, and a 200 to 1 dilution of concentrated ammonium hydroxide (sp. gr. 0.90) until the filtrate came through colorless. The filter pad was scraped, and the residue was suspended in 50% saturated ammonium sulfate at pH 8.0 and refiltered. Subsequent attempts to elute the cytochrome with 0.06 M disodium phosphate (ref. 10) and with other eluting agents were unsuccessful.

The second procedure was that described by Lundegardh (ref. 26). In this method the chloroplast fractions were extracted with 85% acetone. The depigmented protein precipitate was filtered, washed with acetone, resuspended in water, and read directly in the spectrophotometer by using a modification of the technique of Shibata (ref. 33) to measure turbid solutions. Instead of employing opal glass (ref. 33) or filter paper (ref. 26) to equalize light scattering, the 1-cm^2 cuvettes were turned so that their frosted, nonoptical surfaces were in the light path. In this manner, a reasonably flat base line was obtained in the Beckman DK-1 recording spectrophotometer when water was used in both the reference and the sample cells.

The third procedure was a modification of that used by Gross and Wolken (ref. 17). It was modified for lyophilized material by eliminating the digitonin-extraction step. Lyophilized chloroplast material was extracted with 80% acetone to remove pigments; the precipitate, washed and dried in absolute acetone, was treated as the cytochrome-containing residue and was fractionated with ammonium sulphate at 45% and 90% saturation, respectively. Finally, all these fractions, both precipitates and supernatants, were examined spectrophotometrically for the presence of cytochrome.

B. Physiology

1. Electron Transfer

Hill activity was measured by the reduction of ferricyanide, as previously described (refs. 2, 18). Specific activity was recorded as μmoles of electrons

transferred per mg of chlorophyll or protein per hr of illumination. These measurements were performed on chloroplast fractions to establish differences in activity or changes in activity levels related to structural and chemical differences.

2. pH Studies

In order to determine the influence of pH on Hill activity, chloroplast fragments were assayed in different buffers at a number of pH levels and at like chlorophyll concentrations after 8 and 24 hr of storage.

Chloroplast fractions stored under standard conditions (0.015 N sodium chloride) were assayed in 0.05 M phosphate and 0.015 M Tris-maleate buffers. Both buffers contained 0.015 N sodium chloride. The pH levels of the buffers were 5.8, 6.3, 6.8, 7.3, and 7.8. In addition, the Tris-maleate buffer was used at pH 8.3.

3. Supernatant Effect

Chloroplast fractions were suspended in 0.015 N saline, separated into two aliquots, and resedimented at the speed originally used to obtain the fraction. The control aliquot was resuspended in saline, and the other aliquot was resuspended in the supernatant remaining after centrifuging for 30 min at 145,000 x g. The resuspended fractions were periodically tested for Hill activity during a 24-hr storage period under the given conditions.

C. Structure

1. Electron Microscopy

Formvar-coated 200-mesh copper grids were used for mounting specimens. The mounts were prepared according to Hall (ref. 20) by placing a droplet of particle suspension on a dust-free grid, absorbing the excess solution with the edge of a piece of filter paper, and gently washing the preparation with successive microdrops of distilled water. Finally, excess water was withdrawn by the filter paper method and the grid, on a clean glass slide in a petri dish, was dried overnight in a desiccator. In some experiments polystyrene latex particles of uniform, known diameters, 8140 A or 880 A, depending upon the magnification to be used, were mixed with the suspension prior to mounting. Untreated and metal-shadowed specimens were examined. Germanium or alloys of gold and palladium or gold and nichrome were used for metal-shadowing. In some experiments the Hitachi HS-6 microscope was employed. To obtain better resolution, some observations were made with the Hitachi HU-11.

2. X-Ray Diffraction

By using the Debye-Scherrer method, x-ray photographs were taken of concentrated chloroplast fractions in water. The suspensions were drawn into Raebiger 0.3-mm-bore thin-walled capillary tubes or into capillaries

of 1 mm bore drawn from pyrex tubing. One sample from CF_{145s} was used as a dry powder. The capillaries were mounted in a standard Philips x-ray unit and exposed to Cu-K α-radiation of 1.5405 A at 40 kv and 18 mamp. Photographs were taken on Philips 5.73-cm-radium cylindrical powder cameras.

3. Electrophoresis

Chloroplast fractions were subjected to electrophoresis in a pH gradient according to the method of Kolin (ref. 24). The electrophoresis cell was a pyrex U-tube with side chambers for the electrodes and was activated by a standard power supply, the Hewlett-Packard model 711 A (fig. 3). Interesting and relatively promising results were obtained with this rather crude arrangement.

The experimental procedure was to fill the U tube to one third of its height with acid buffer, 50% saturated with sucrose, with a syringe and large gauge needle. To the sample suspension, which had been dialyzed 3 hr in the cold against distilled water, was added 10 to 25% sucrose, depending upon the experiment. This was layered above the acid-sucrose buffer in the right-hand arm of the electrophoresis cell. The height of the sample layer was equal to the inside diameter of the tube, as recommended by Kolin. Above the sample layer and in the adjoining electrode chamber sucrose-free alkaline buffer was dispensed. The opposite arm and its electrode compartment (anode) were filled with sucrose-free acid buffer; the cathode, then, was in contact with the alkaline buffer.

The buffer systems used were either Michaelis' universal veronal-acetate buffer (ref. 24) or citrate-phosphate buffer prepared according to Gomori (ref. 13). Sodium chloride was added to the buffers when needed to increase the ionic strength. In general, the ionic strength was adjusted so that the current could be maintained at about 5 mamp, thus permitting separations in about 5 min. At the end of a run, separated fractions were isolated by means of a 5-ml syringe with a long 18- or 20-gauge needle bent to 90° at the tip to facilitate withdrawal with minimal disturbance of the column.

4. Ultracentrifugal Analyses

Chloroplast fragments were prepared by the standard procedure and analyzed in the diluting medium in which they were isolated. For CF_{20-50} and CF_{70-145}, the diluent was 0.015 N sodium chloride. The supernatant solution, CF_{145s}, was essentially in the same diluting solvent but had about 0.04 M sucrose and 0.005 M phosphate buffer remaining from the original chloroplast isolation medium. Prior to study in the Beckman model E ultracentrifuge, it was calculated that CF_{20-50} would have to be analyzed at less than 20,000 rpm and CF_{70-145} at near 20,000 rpm (32,000 x g). Therefore, to remove large aggregates, low-speed preparative centrifugation in an International PR-2 centrifuge at 4 °C at 2,500 x g was used for CF_{20-50} and CF_{70-145} for 10 min and 15 min, respectively. CF_{145s} was precentrifuged at 6000 x g for 15 min in a Servall model SS-1 centrifuge in a 4 °C cold room. For analysis, the rotor was kept at approximately 20 °C.

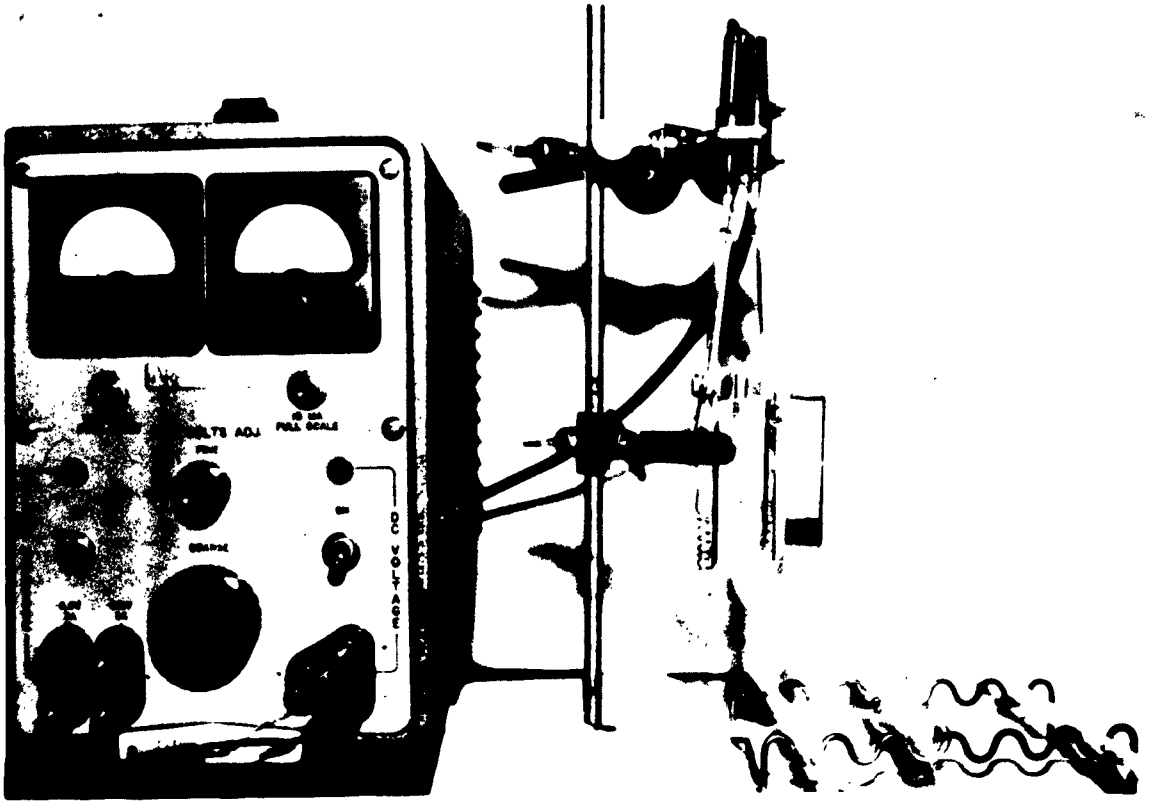


Figure 3

APPARATUS USED FOR ELECTROPHORESIS

Because of problems of light absorption by chlorophyll, especially during analysis of CF_{20-50}, the Schlieren light source slit was opened 3/8 of a turn and/or the Wratten 77A filter was removed from the optical path as necessary. When chlorophyll was at a concentration greater than 0.5 mg/ml in CF_{20-50}, the light absorption was so high that poor photographic reproduction of the Schlieren pattern was obtained. When the chlorophyll concentration was less than 0.25 mg/ml, the protein concentration was so low that a poor Schlieren pattern resulted. Therefore, for all analyses of chloroplast fractions, the chlorophyll concentration was maintained between 0.25 and 0.5 mg.

Samples were analyzed in a 3-mm aluminum centerpiece and a small phase-plate angle was used. CF_{145s}, which was low in chlorophyll content and relatively high in protein content, presented no special problems and was analyzed in a 12-mm centerpiece with no modification of the standard analytical ultracentrifugation technique.

IV. EXPERIMENTAL RESULTS AND DISCUSSION

A. Chemistry

1. Chlorophylls

The activity differences between differentially centrifuged particulate fractions of isolated chloroplasts were previously shown to be correlated with chlorophyll-to-protein ratios (ref. 18). In the current work measurements of chlorophyll and protein in relation to dry weight showed that the amount of chlorophyll remained relatively constant from fraction to fraction.

Since more than one kind of chlorophyll exists, it was of interest to determine whether the ratio of one type of chlorophyll to another might change in relation to activity. The extinction ratios of the long-wavelength maxima of chlorophyll a and chlorophyll b in the various chloroplast fractions were compiled from several experiments, and the ratio ranges are recorded in Table 1. Ratio changes are apparent, but their significance is not yet conclusively established.

Table 1

RANGE OF RATIOS OF EXTINCTIONS OF CHLOROPHYLL a AT 663 mμ AND CHLOROPHYLL b AT 645 mμ IN 80% ACETONE

Fraction	No. of Expts.	E_{663}/E_{645}
CF_{os}	9	2.54-2.62
CF_{1-20}	6	2.61-2.68
CF_{20-50}	8	2.68-2.76
CF_{70-145}	3	2.90-3.07
CF_{145s}	3	2.80-2.84
$CF_{145-173,30}$*	2	2.69-2.70
$CF_{173,30-173,60}$*	1	2.98
$CF_{173,60-173,120}$*	1	1.33

*These fractions were serially centrifuged at 173,000 x g from CF_{145s} at 30, 60, and 120 min as indicated.

It was believed that more critical measurements might divulge significant information about the ratio differences. In collaboration with Dr. W. L. Butler of the U.S. Department of Agriculture, exploratory studies of a series of similar fractions were made by low-temperature absorption and fluorescence spectrophotometry. These experiments established that the long-wavelength chlorophyll C-705 (ref. 6) was present in all chloroplast fractions in addition to chlorophylls a and b. A representative curve demonstrating the three chlorophylls is presented in Figure 4. While ratio changes are not readily discernible, it should be noted that the spectral data were never completely analyzed.* In view of the data in Table 1, especially those for the CF_{173} set of fraction, it would seem desirable to investigate this aspect further in the future.

2. Protein and Lipid

The total nitrogen content of chloroplast fractions was measured, and protein nitrogen was calculated by substracting the contribution of the nonprotein nitrogen content of chlorophyll from the total (ref. 18). Although nonprotein nitrogen other than chlorophyll nitrogen is present in chloroplasts in equal concentration to chlorophyll nitrogen (ref. 30), it can be disregarded in calculating protein nitrogen since only a 5 to 6% correction would be necessary. Such a correction would introduce a negligible change in chlorophyll-to-protein ratios in WC, CF_{Os}, and CF_{145s}. No correction would be required in the sedimented CF fractions since most, if not all, of the nonchlorophyll, nonprotein nitrogen would have been leached out during sonication, washing, and suspension in hypotonic saline. The protein content was then calculated from the amount of protein nitrogen present. These data were related to dry weights, as indicated in Section 3 below. The difference between the measured dry weight and the calculated weight of chlorophyll and protein is ascribed chiefly to the weight of the lipids in the assayed fraction and is referred to as the "lipid fraction." The contribution of nonprotein nitrogen components to the weight of the "lipid fractions" of CF_{Os} and CF_{145s} would be expected to be small since these components would be chiefly low molecular weight compounds which would have been removed by the dialysis procedure used (see Section 3, below).

3. Dry Weights

Data on the ratios of chlorophyll to protein in the isolated chloroplast fractions led to the question of which chemical components were changing in concentration from one fraction to the next. Therefore, the actual weights of the components in relation to the total weight of the fraction had to be determined.

Since the isolation medium for whole chloroplasts had a high level of sucrose and salts, the contribution that these would make to the weight of a fraction, even when diluted 100-fold with distilled water, would be a minimum of 1.55 mg/ml. CF_{Os} preparations contained higher amounts of sucrose and salts, which might add as much as 4.0 mg/ml of nonsample weight. A similar level

*Personal communication from Dr. Butler.

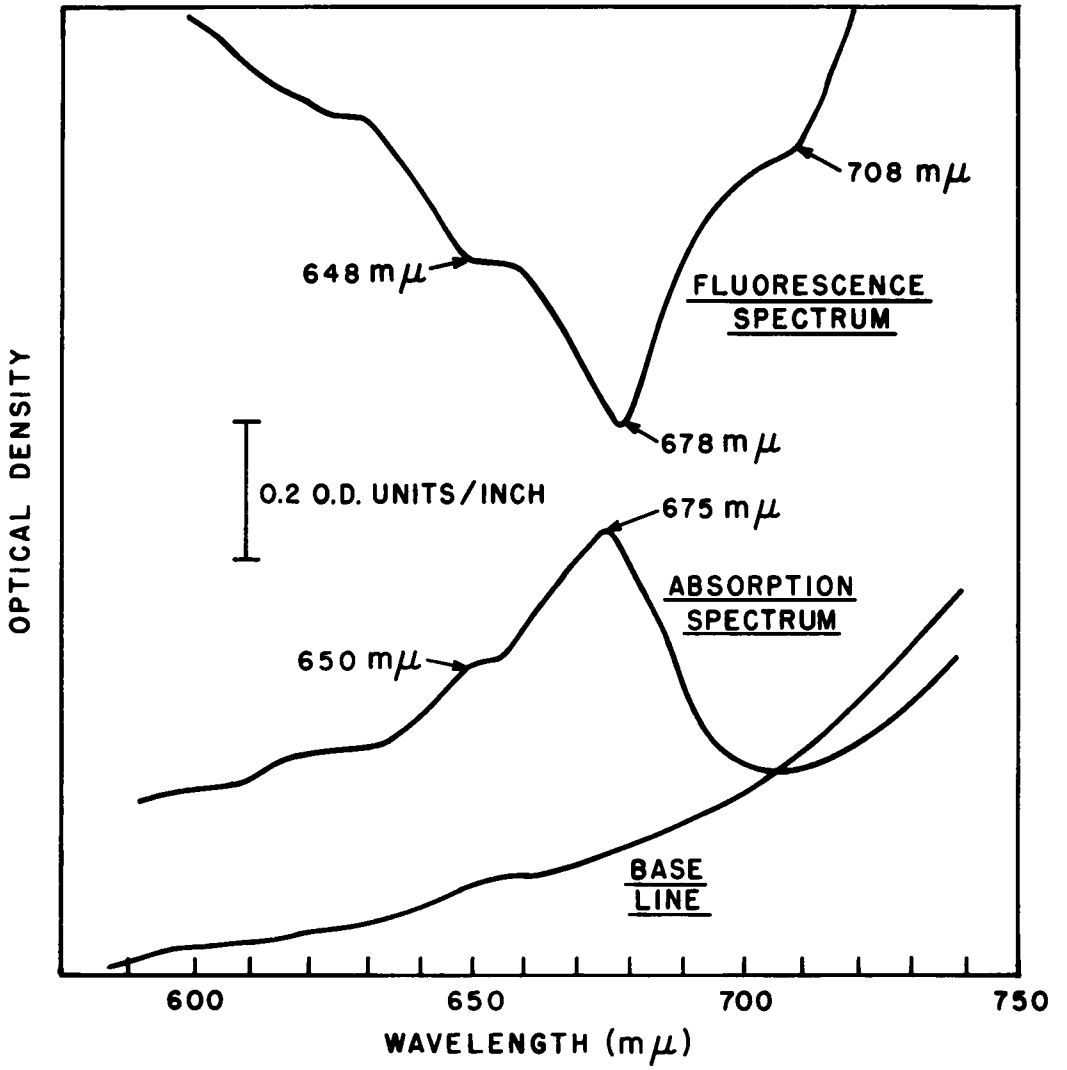


Figure 4

LOW TEMPERATURE (-196°C) ABSORPTION AND FLUORESCENCE SPECTRA OF AQUEOUS SUSPENSIONS OF CF_{20-50}

of excess material would be found in CF_{145s}. Consequently, the elimination of these constituents was essential to accurately measure dry weights of the fractions. The techniques of dialysis or of centrifugation and washing surmounted these difficulties. The percentage of chlorophyll, protein, and lipid fraction on a dry weight basis for the various fractions is recorded in Table 2.

Table 2

COMPOSITION OF CHLOROPLAST FRACTIONS

Fraction	Percent of Total Dry Weight		
	Chlorophyll	Protein	Lipid Fraction*
CF_{os}	6	52	42
CF_{1-20}	10	58	32
CF_{20-50}	10	51	39
CF_{50-70}	10	66	24
CF_{70-145}	8	69	23
CF_{145s}	0.5-1.0	45	54

*Determined by difference.

The chlorophyll to protein ratios calculated from dry weights (table 2) and those based on direct measurements of these two components (ref. 18) are compared in Table 3. There is relatively good agreement between the two methods.

4. Plastoquinone

a. Quantitative Determination and Relations

The importance of PQ in photosynthesis has been suggested by the researches of a number of investigators (refs. 5, 9, 25, 31, 36). The PQ content of isolated particulate fractions of chloroplasts have been determined and related to other components and Hill activity of the fractions (ref. 3). In general agreement with the findings of Crane (ref. 8) and Redfearn and Friend (ref. 31), the mole ratio of PQ to chlorophyll in WC and CF_{os} was determined as 0.07 and the number of μmoles of PQ per mg of protein was found to be 9.0. Analyses of the chloroplast fractions are shown in Table 4.

Table 3

COMPARISON OF CHLOROPHYLL-TO-PROTEIN RATIOS

Fraction	Ratios	
	Chemical Assay (ref. 18)	Chemical Assay and Dry-Weight Measurements (Table 2)
CF_{os}	0.121	0.115
CF_{1-20}	0.251	0.170
CF_{20-50}	0.201	0.187
CF_{50-70}	0.157	0.153
CF_{70-145}	0.066	0.123
CF_{145s}	0.0077	0.0100

Table 4

RELATIONSHIP OF CHLOROPHYLL, PQ, AND PROTEIN TO HILL ACTIVITY

Fraction	μmoles per mg of protein		Mole Ratio of PQ to Chlorophyll	Activity μmoles electrons transferred per hr per mg of chlorophyll
	PQ	Chlorophyll		
WC	9.0	130	0.07	30
CF_{os}	9.5	135	0.07	102
CF_{20-50}	22.6	214	0.11	155
CF_{70-145}	10.0	104	0.10	55
CF_{145s}	1.3	7.14	0.18	25

When the PQ in the various fractions is compared with protein rather than with chlorophyll, the changes are significant. WC, CF_{os}, and CF_{70-145} have similar amounts of PQ and chlorophyll per mg of protein but differing activities. CF_{20-50} has the greatest quantity of these constituents and the highest activity. Finally, the smallest amount of either PQ or chlorophyll per mg of protein and the lowest activity is in CF_{145s}.

Yet, it is not fair to compare the activities of sedimented fragments (CF_{20-50} and CF_{70-145}) with intact or broken chloroplasts (WC and CF_{os}), nor with the supernatant, CF_{145s}. WC and CF_{os}, which are chemically alike are structurally different. They contain much nonphotoactive material not present in CF_{20-50} and CF_{70-145}. Likewise, CF_{145s} contains an abundance of nonphotoactive constituents and relatively few, small photoactive particles.

Nevertheless, correlation of the chemical composition with Hill activity (Table 4) suggests a functional interaction among the constituents. While the relationships are neither simple nor direct, since other factors, such as spatial orientation of the molecules, also play a role in photosynthetic efficiency, correlation is evident, albeit a nonlinear type.

b. Reconstitution with PQ

The PQ extraction method used for PQ analysis was aimed at obtaining a quantitative yield. Along with PQ, other lipid-soluble components, including a major portion of the chlorophyll pigments, were extracted. Therefore, for the reconstitution studies a solvent which would extract a major portion of the PQ and as little as possible of other lipid-soluble components was preferred. Extraction with petroleum ether (b. p. 30-60 °C) as described by French (ref. 12 and Bishop (ref. 5) was utilized.

Experiments were performed according to the outline described in Fig. 2. The isopropanol-heptane extracted PQ fraction which had been stored as a pool was used as the source of PQ for readdition to the residues of fractions extracted with petroleum ether. WC and CF_{20-50} were used in these experiments. The study of WC paralleled, in part at least, the research of others (ref. 5, 25) in that inhibition accompanied extraction and some recovery of activity was realized when PQ was added. WC therefore served as a control and criterion of the efficacy of the technique. CF_{20-50} was selected because it was the fraction with the highest Hill activity and greatest stability and therefore was expected to be the most sensitive fraction for detecting activity changes.

WC and CF_{20-50} were prepared from the same batch of spinach leaves and were assayed simultaneously. Appropriate controls were used to denote the effect of storage time, lyophilization, extraction, and resuspension. WC was stored in 0. 35 M sodium chloride instead of the sucrose-phosphate buffer generally used, in order to elicit a higher Hill reaction rate (ref. 18). It was felt that changes in activity could then be more readily evaluated.

Extraction of WC with petroleum ether resulted in 38% inhibition of Hill activity, while CF_{20-50} was almost totally inhibited. The addition of 0.1 mole PQ per mole chlorophyll to the extracted preparations failed to increase the activity of CF_{20-50} but induced a 30% recovery in WC. These data are recorded in Table 5.

Table 5

RECONSTITUTION OF WC AND CF_{20-50} WITH PQ FRACTION

Fraction Conditions	Activity*		Percent of Control Activity	
	WC	CF_{20-50}	WC	CF_{20-50}
Fresh (control)	397	130		
Stored 24 hr	316	146	80	112
Stored 48 hr	300	160	76	123
Lyophilized and resuspended	175	192	44	148
Lyophilized and resuspended (control)	175	192		
Lyophilized, extracted, and resuspended	108	4.6	62	2.4
Lyophilized, extracted, PQ added, and resuspended	140	4.2	80	2.2
Lyophilized, extracted, and resuspended (control)	108	4.6		
Lyophilized, extracted, PQ added, and resuspended	140	4.2	130	91

*μmoles electrons transferred per mg of chlorophyll per hr.

After extraction with petroleum ether a uniform aqueous suspension of the WC residue was readily obtained; CF_{20-50} could not be readily or uniformly resuspended. This difference might be partly responsible for its failure to react. Other explanations for the differential response observed can be advanced, but none have been tested yet.

While other investigators also have been able to show stimulated activity by readdition of PQ to extracted chloroplast systems, none have demonstrated 100% recovery. Obviously a variety of physical forces influence readsorption of the added components. It is likely that these forces prevent the reorganization of the molecules exactly as they were in the original active system. These problems appear to be amplified for fragment systems like CF_{20-50}.

5. Cytochrome

Although digitonin extraction (ref. 17) previously failed to yield the chloroplast cytochrome f from spinach (ref. 18), the presence of this cytochrome in spinach has been established (refs. 10, 21). Therefore, a further effort to extract the cytochrome from chloroplast fragments was made. The effort described here was aimed at determining whether most of the cytochrome was water-soluble or particle-bound. It was reasoned that if it were soluble, the cytochrome would be found in the supernatant of the 145,000 x g centrifugation, CF_{145s}, since it would leach out of the whole chloroplasts, WC, when they were subjected to sonic treatment. On the other hand, if most of the cytochrome were particle-bound, it would be found in the sedimented fractions of the fragmented chloroplasts.

Davenport and Hill's (ref. 10) method of detecting cytochrome gave qualitative evidence of the presence of a cytochrome in sedimented chloroplast fragments (CF_{1-50}) but not in CF_{145s}. All attempts to elute the reddish cytochrome-like precipitate which adsorbed to the kieselguhr failed. The eluting solutions used were 0.06 M disodium phosphate, as recommended (ref. 10), 0.05 M ammonium carbonate, citrate buffer (pH 5.2), Coleman pH buffer (pH 4.0), Beckman pH buffer (pH 7.0), Sorensen phosphate (pH 6.8), Tris-hydrochloride (pH 8.1), 100% ethanol, 80% ethanol, and 40% ethanol.

Lundegardh's method (ref. 26) showed cytochrome peaks identifiable by spectrophotometry in sedimented depigmented CF_{1-50} but not in CF_{145s}. The cytochrome content, however, was too low to make a quantitative estimate.

That cytochrome was present in chloroplast fragments was ascertained by the above two methods. The modified Gross and Wolken method (ref. 17) was applied only to CF_{145s} to verify the absence of soluble cytochrome. A measurable amount of cytochrome could not be demonstrated.

While none of the experiments performed is definitive, it seems clear that most of the cytochrome must be particle-bound (CF) and little, if any, is in the soluble form (CF_{145s}). In the chloroplast then, the cytochrome must be in a molecular complex, probably associated with chlorophyll and not free in the stroma.

B. Physiology

1. Electron Transfer

Electron transfer was measured by the standard method unless otherwise specified. Table 6 lists average values of specific activities of chloroplast fractions after 8 and 24 hrs of storage.

Table 6

SPECIFIC ACTIVITY OF CHLOROPLAST FRACTIONS AFTER 8 AND 24 HR OF STORAGE

Fraction	Electrons Transferred per mg of chlorophyll		Electrons Transferred per mg of protein	
	8 hr	24 hr	8 hr	24 hr
CF_{os}	101	89	11.9	10.5
CF_{0-1}	74	85	12.0	13.9
CF_{1-20}	119	135	24.0	27.2
CF_{20-50}	141	167	27.4	32.5
CF_{50-70}		81		12.6
CF_{70-145}	46	51	4.3	4.8
CF_{145s}	28		0.25	

2. pH Studies

Preliminary studies of electrophoretic separations from the differentially centrifuged chloroplast fractions revealed that most of the chloroplastic material had an isoelectric point at or below pH 5.8. It was therefore of considerable interest to determine whether the Hill reaction could proceed at this pH prior to establishing differential activities in the electrophoretically separated fractions.

Previous studies in this program employed an isolation medium of unbuffered 0.015 N sodium chloride and an assay system of 0.05 M phosphate buffer (pH 6.8) containing 0.015 N sodium chloride. The selection of these solutions had been based on prior experimentation and information from the literature. It had also been noted that the fractions responded to changes in the concentration of sodium chloride and phosphate buffer (ref. 18). In view of these data, a study of the role of pH was deemed essential.

The most active fraction, CF_{20-50}, and the less active, CF_{70-145}, were used. The results of assays on both fractions in Tris-maleate and phosphate buffers are summarized in Figure 5. Except for the results at pH 5.8 in phosphate, for which there seems to be no simple explanation, activity was always higher in CF_{20-50} than in CF_{70-145}. As a matter of fact, after 24 hr of storage under the experimental conditions, the activity of CF_{20-50} at pH 5.8 became the greater of the two, as it was at all other pH levels.

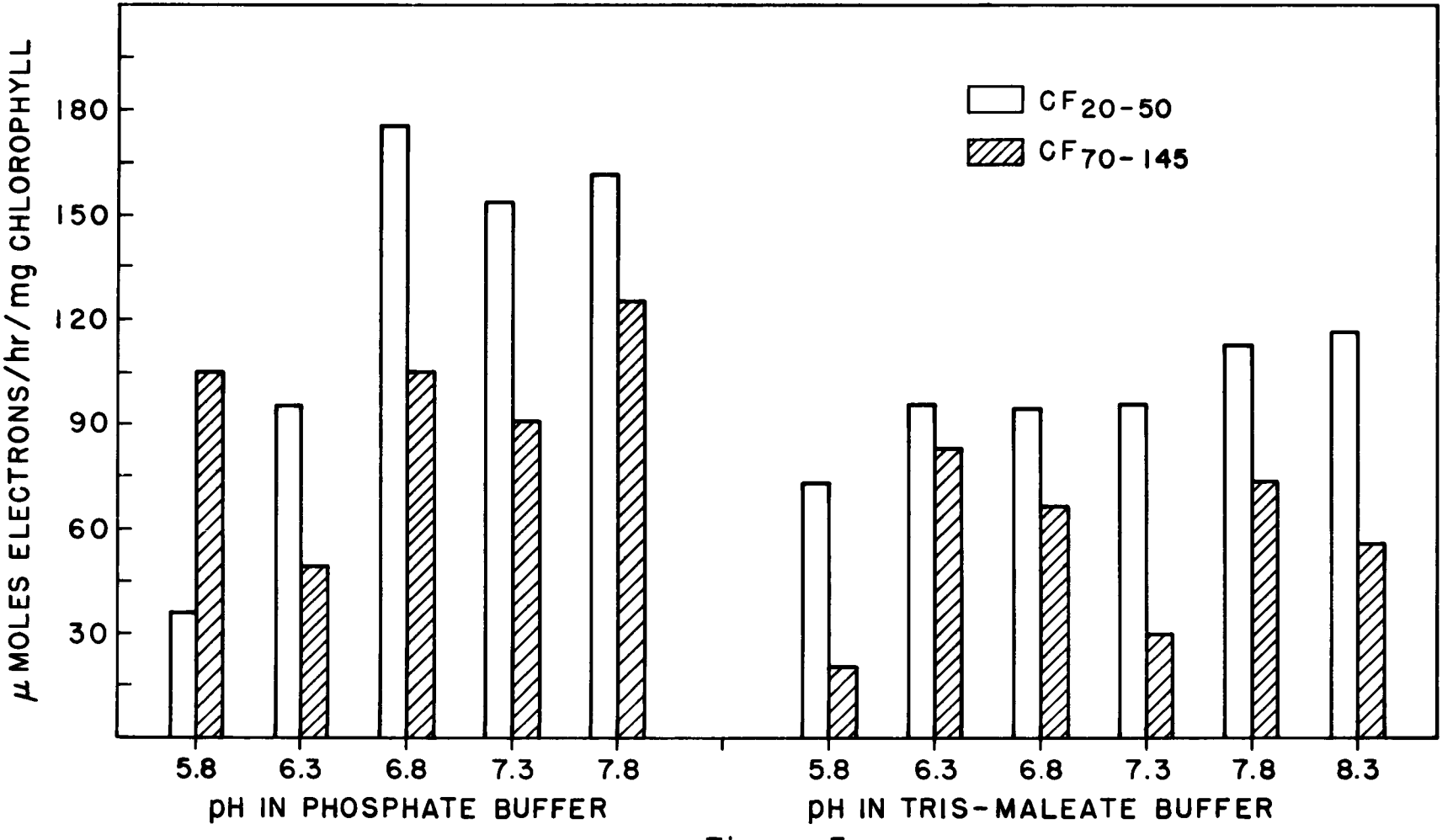


Figure 5

THE EFFECT OF pH ON CF_{20-50} AND CF_{70-145} AFTER 8hr STORAGE

It can also be seen that phosphate is the preferred buffer in comparison with Tris-maleate. Good (ref. 15) has shown that 0.01 to 0.03 M Tris-hydrochloride is more inhibitory than Tricine (a glycine derivative of Tris). In addition, Good has shown that Hill activity of whole chloroplasts isolated in Tris-hydrochloride at pH 7.5 was variable in the presence of phosphate, sulfate, citrate, or lactate. The response did not show a direct relationship to the concentration of the different anions.

Other investigators studying the effects of parameters such as pH and salt concentrations have found variable responses for whole and broken chloroplasts. Kandler and Elbertzhagen (ref. 22) demonstrated a pH dependence of carbon dioxide fixation by whole and broken chloroplasts, the optima being between pH 7.0 and 7.5, and pH 8.0 and 8.5, respectively. Quantitative results in the pH studies reported here were also variable, the same conditions not necessarily being optimal for both CF_{20-50} and CF_{70-145}. Vennesland and Stiller (ref. 35) and Good (ref. 14) have also reported that the medium used for chloroplast isolation affects both Hill reaction rate and photophosphorylation. These reports tend to confirm the variable influence of pH on photosynthetic reactions.

To summarize, it was ascertained that for reliability of experimental results it was desirable to isolate and store fractions in unbuffered 0.015 N sodium chloride and assay in phosphate buffered sodium chloride at pH 6.8.

3. Supernatant Effect

Hill activity of CF_{20-50} was previously shown to be inhibited by a 24-hr exposure to CF_{145s}; the inhibition was 40% greater than when stored in 0.015 N sodium chloride for the same period of time (ref. 18). In the current work it was found that exposure of CF_{70-145} to CF_{145s} for 24 hr stabilized Hill activity, so that it was twice that of the same fraction stored in 0.015 N sodium chloride solution although the activity declined under both storage conditions (table 7).

Table 7

EFFECT OF CF_{145s} ON HILL ACTIVITY OF CF_{70-145}

Storage Medium	Activity, μmoles electrons transferred per mg of chlorophyll per hr	
	After 4 hr	After 24 hr
0.015 N saline	110	48
CF_{145s}	120	90

The significance of these observations is uncertain at this time. If these responses reflect reassociations or disassociations of chloroplast fragments in the presence of supernatant components they can be studied by experimental analytical ultracentrifugation. Studies of this nature have not as yet been performed.

C. Structure

1. Electron Microscopy

Figures 6 through 9 are representative electron micrographs of CF_{os}, CF_{20-50}, CF_{70-145}, and CF_{145s}, respectively. Polystyrene latex particles of known dimensions were included in the fragment suspension to facilitate measuring particle sizes directly from the photographs. From these figures and a number of similar photographs, the dimensions of the chloroplast fragments found in each fraction were measured. The data are shown in Table 8.

Table 8

DIMENSIONS AND DISTRIBUTION OF FRAGMENTS
IN DIFFERENTIALLY CENTRIFUGED CHLOROPLAST FRACTIONS

Fraction	Parameter	Fragment Diameter, A	Fragment Height, A	Ratio of Mean Diameter to Mean Height
CF_{os}	Range	200-18250	100-1600	
	Mean	2500		
	Median			
	Mode			
CF_{20-50}	Range	500-6750	200-1680	
	Mean	1500	360	
	Median	950	250	4.35
	Mode	950	250	
CF_{70-145}	Range	350-2500	250-600	
	Mean	500	352	
	Median	450	300	1.36
	Mode	450	300	
CF_{145s}	Range	200-350	100-350	
	Mean			
	Median			approx. 1
	Mode			

14600 X

34000 X

Figure 6

ELECTRON MICROGRAPHS OF GOLD-NICKEL SHADOWED CF_{os}
(Arrow indicates 880 A PSL)

8000 X

27800 X

Figure 7

ELECTRON MICROGRAPHS OF GERMANIUM SHADOWED CF_{20-50}
(Arrow indicates 8140 A PSL)

13400 X 16000 X

Figure 8

ELECTRON MICROGRAPHS OF GERMANIUM SHADOWED CF_{70-145}
(Arrow indicates 880 A PSL)

26000 X 45500 X

Figure 9

ELECTRON MICROGRAPHS OF CF_{145s}
(Arrow indicates 880 A PSL)

A comparison between these data and those reported previously (ref. 18) shows fair agreement between average size estimates from sedimentation data and measurements from electron micrographs. The data from CF_{145s} compare fairly well with the measurements reported by Thomas et al. (ref. 34), except that the size range of his particles was from 30 A in diameter to more than 330 A. Compare these values with those in Table 8. It should be noted that Thomas' methodology was different. Table 8 also shows that there is greater variability in the mean height of larger particles (CF_{20-50}) than of smaller particles (CF_{70-145}). The ratio of mean diameter to mean height suggests a discoid or oblate spheroid shape for all the fragments. The smaller fragments are more nearly spherical, as indicated by the approach of the ratio to unity.

2. X-Ray Diffraction

Although there was little optimism that Debye-Scherrer photographs would show more than halo diffraction patterns which are normal for amorphous materials, it was considered worthwhile to attempt to find some characterizing property. All the liquid samples showed the broad halo patterns expected. These seemed to correlate with the patterns of water and the wall thickness and diameter of the capillaries used. The dry powder, which was a green acetone precipitate from CF_{145s}, showed a line pattern for the dihydrate of calcium oxalate and another unidentified crystalline material in addition to the halo pattern. Oxalic acid and calcium salts are common constituents of plants and are especially abundant in spinach. Crystals of calcium oxalate are often observed in the vacuoles of plant cells. Consequently, it was not surprising that the salt was observed in the x-ray pattern.

Since the longest spacing observable by the x-ray technique employed is about 15 A, the standard Debye-Scherrer method is of limited value. The intrinsic spacing sin an organized unit might be expected to be similar to those of the chloroplast. The smallest repeating spacing in the chloroplast is known from electron microscopy to be about 30 A (refs. 18, 28). Therefore, it is proposed that a flat cassette transmission technique be used in which the film-to-specimen distance is greatly increased, thus effectively magnifying the diameter of the diffraction image to permit longer spacings to be observed.

3. Electrophoresis

These studies were conducted to explore the potential value of electrophoresis in a pH gradient for (1) separating particulate fractions from the CF_{os} mixture to compare with the differentially centrifuged fractions obtainable from CF_{os}, and for (2) determining the chemical-structural homogeneity of the centrifuged fractions. It was reasoned that fragments sedimented on the basis of size might not be chemically uniform, especially since the structure from which they were originally derived, the intact spinach chloroplast, is structurally heterogeneous. If this rationale were correct, it might even be possible to isolate from a centrifugal fraction such as CF_{20-50} a chemically and structurally pure fraction which might be responsible for the high Hill activity of the parent mixture and would itself exhibit an even higher reaction rate. Consequently, the potential value of this method had to be experimentally determined by:

(1) Electrophoresis of the chloroplast fractions and isolation of any fractions which might separate from them

(2) Determination of the activity level of the electrophoretically isolated fractions

(3) Chemical and/or structural characterization of the isolated fractions.

Chloroplast fractions for electrophoretic separation were dialyzed against an 8-liter volume of cold distilled water for 3 hr at an exchange rate of 2 liters per hr. They were further dialyzed overnight against 40 ml of distilled water to stablize the system. Thus, electrolytes which would interfere with electrophoretic migration of the fragments were eliminated. In order to determine the effect of this preparatory method, fractions were examined spectrophotometrically and assayed for Hill activity before and after dialysis. The results of these experiments are shown in Tables 9 and 10, respectively.

Table 9

COMPARISON OF ABSORPTION PEAK RATIOS
OF CHLOROPLAST FRACTIONS BEFORE AND AFTER DIALYSIS

Condition	Fraction	E_{437}/E_{678}	E_{262}/E_{678}	E_{262}/E_{270}
Before dialysis	CF_{os}	1.77	1.48	1.24
	CF_{1-20}	1.79	1.70	1.04
	CF_{20-50}	1.75	1.43	1.03
	CF_{70-145}	1.80	2.23	1.03
	CF_{145s}	1.90	6.48	0.92
After dialysis	CF_{os}	1.76	1.70	1.04
	CF_{1-20}	1.76	1.79	1.04
	CF_{20-50}	1.73	1.55	1.03
	CF_{70-145}	1.87	2.65	1.04
	CF_{145s}	1.91	6.12	0.96

Table 10

HILL ACTIVITY OF CF_{20-50} BEFORE AND AFTER DIALYSIS

Condition	Assay Solution	Activity μmoles electrons transferred per hr per mg of chlorophyll After 0 Hr	After 3 Hr	After 24 Hr	After 48 Hr
Before dialysis	Standard	159	148	132	141
	Standard plus 0.06 M sucrose		160	126	144
After dialysis	Standard		153	136	130
	Standard plus 0.06 M sucrose		123	153	150

E_{678} represents the extinction of the red peak of chlorophyll a (678 mμ) in aqueous suspension. E_{437} represents the blue peak of chlorophyll a (437 mμ) (ref. 30) and includes carotenoid absorption. In all fractions except CF_{145s} there was an absorption maximum at 262 mμ (E_{262}) which is interpreted as representing the combined absorption of nucleoproteins and other proteins. E_{270} generally reflects the absorption of proteins other than nucleoproteins. The ratios of these absorption peaks reflect the relative amounts of the represented components and can be used as indices of differences. Thus, the ratio of E_{437}/E_{678} indicates the carotenoid-chlorophyll a relationship, since the blue and red peaks of chlorophyll a can be expected to change in direct proportion to each other. E_{262}/E_{678} represents the total protein-chlorophyll a change, and E_{262}/E_{270} indicates the relationship between nucleoproteins and nonnucleoproteins.

From Table 9 it can be seen that dialysis does not appreciably change these ratios, although there are differences from fraction to fraction, some of which confirm previously reported data demonstrating chemical differences (ref. 18). For example, the low E_{262}/E_{678} ratio of CF_{20-50} reflects a relatively small amount of protein in relation to chlorophyll. Likewise, the high ratio in CF_{145s} reflects a relatively large amount of protein per unit of chlorophyll. The E_{437}/E_{678} ratio in CF_{145s} suggests a relatively larger amount of carotenoid material than in other fractions. Likewise, the drop in the E_{262}/E_{270} ratio may be significant. Further experimental evidence is required before any conclusions can be made about these differences.

The activity measurements of CF_{20-50} (table 10) indicate that dialysis, the time of storage, and the presence of sucrose in the assay medium do not have a significant effect on activity. These data indicate that preparation of samples for electrophoresis by dialysis and dilution with sucrose solution would have no detrimental effect on activity prior to the electrophoretic fractionation.

Fig. 10 depicts the results of electrophoretic fractionations of the chloroplast fractions. The sample column in the electrophoretic cell at the beginning of an experiment (Fig. 10 a) and after 3 min at a current of 5 ma (Fig. 10 b) is shown.

CF_{os} was found to separate into two to three bands in Michaelis' buffer, the sharpest fractionation occurring in the pH range 3.0 to 5.7 (Fig. 10 c). The pH ranges 3.8 to 9.8 and 3.8 to 7.0 gave less clear-cut separations. In the pH range of 3.0 to 5.7 the third band (γ) was diffuse, more anodic (acidic) than the rest, and overlapped the second band (β). A fourth band, intermediate in position between α and β, was visible in some experiments.

CF_{1-20} also separated sharply in Michaelis' buffer at pH 3.0 to 5.7, (Fig. 10 c). The effect of concentration of CF_{1-20} in terms of μgrams of chlorophyll per ml of sample was tested. In citrate-phosphate buffer between pH 3.0 and 5.7 when the chlorophyll concentration was 160 μg/ml, only two bands appeared, while at approximately half this concentration of chlorophyll three electrophoretic fractions were observed. However, the separation was not as sharply defined as in Michaelis' buffer.

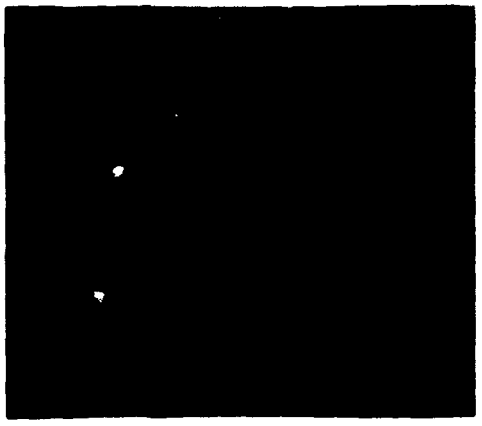
The citrate-phosphate buffer system in the pH range 3.0 to 7.0 yielded the sharpest fractionation of CF_{20-50} (Fig. 10 d). It was found that the concentration of suspended material had an effect on the degree of electrophoretic separation. Levels of 18, 30, and 60 μg of chlorophyll per ml were tested. The best separation was achieved at the lowest concentration of sample. The ionic strength of the buffers also influenced the separations, values between 0.12 and 0.17 being most effective.

CF_{70-145} separated into three distinct fractions in citrate-phosphate between pH 3.0 and 5.7 (Fig. 10 d). CF_{145s} also separated as three bands in this buffer system but two of the bands overlapped at the alkaline side of the column (Fig. 10 d).

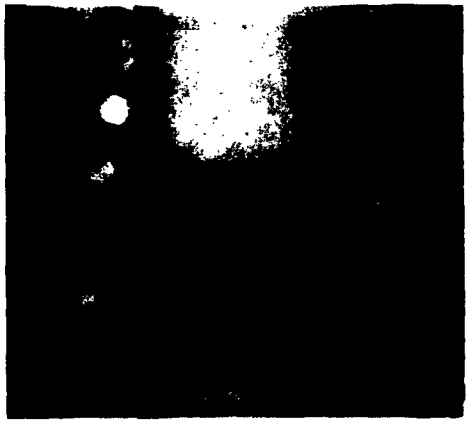
The Hill activity of several of the electrophoretically separated fractions were tested after withdrawal from the column. Generally, the volume of the fraction isolated was so small that no determinations were possible. CF_{20-50}, however, yielded sufficient quantities for measurement. All three fractions, α , β, and γ , showed some Hill activity. Unfortunately, activities could not be quantified because there was insufficient material for assay of both the Hill reaction and the chlorophyll content.

Preliminary investigations of electrophoretic fractions for spectral changes were carried out. No conclusive data were obtained.

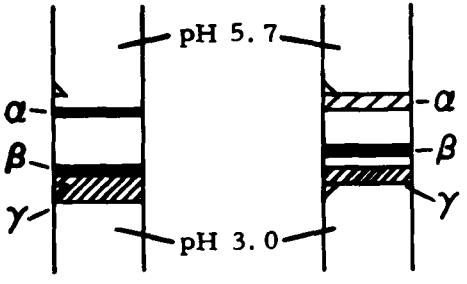
The isoelectric points of the electrophoretically fractionated samples are indicated by the pH value at which they were isolated. These values compare with isoelectric pH's for chloroplastic material of 3.7-4.7 reported in the literature (ref. 30). The Beckman model G pH meter was used to measure pH when sufficient volume was available and narrow-range pH papers were used for small



(a) Sample column before separation.



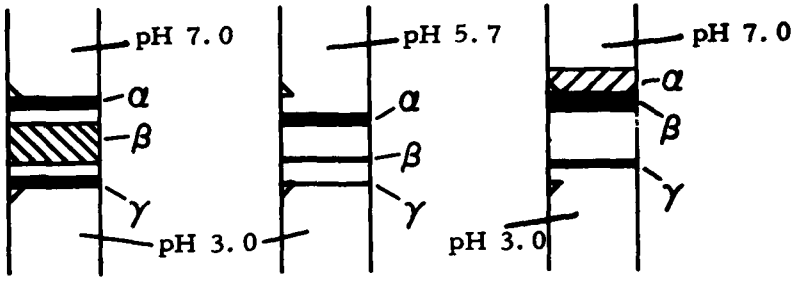
(b) Sample column after separation: 3 min, 5 mamp, CF_{20-50}, pH 3.0-5.7.



CF_{os}
15 mamp
3 1/2 min

CF_{1-20}
11 mamp
5 min

(c) In Michaelis' buffer.



CF_{20-50}
30 mamp
5 min

CF_{70-145}
5 mamp
5 min

CF_{145s}
5 mamp
5 min

(d) In citrate-phosphate buffer

Fig. 10

ELECTROPHORETIC SEPARATIONS OF CHLOROPLAST FRACTIONS

samples (table 11). These data can be compared with the band positions in each fraction from Fig. 10 c and d. The conclusions which can be drawn from these data are that centrifugally differentiated fractions of broken chloroplasts are probably not chemically homogeneous, since they can each be separated into several subfractions at their respective isoelectric points in an electrical field with a pH gradient. The differences in the isoelectric point might also reflect structural changes, since the method is based on surface charge. While there is no absolute evidence for chemical differences, the absorption peak ratios are suggestive of such a change.

Table 11

ISOELECTRIC POINTS OF ELECTROPHORETICALLY SEPARATED CHLOROPLAST FRACTIONS

Fraction	Band	Isoelectric pH
CF_{os}	α	5.4
	β, γ	3.7
CF_{1-20}	α	5.6
	β	
	γ	3.2
CF_{20-50}	α	6.6
	β	4.5
	γ	3.7
CF_{70-145}		Not measured
CF_{145s}		Not measured

It can be concluded that the method is potentially capable of producing useful information about the chloroplast fragments. However, in order to employ the technique optimally, several improvements must be made:

(1) A larger, more critically designed cell which will permit the fractionation of sufficient material for quantitative physiological, structural, and chemical assay is required.

(2) An improved method is essential for stabilizing the position of and isolating the separated fractions after a run is completed. The potential value of aqueous semisolid supporting columns such as agar or nonaqueous greases should be explored.

4. Ultracentrifugal Analyses

The technique of differential centrifugation from a heterogeneous chloroplast fragment mixture, CF_{os}, yields more homogeneous particle size fractions. The uniformity of particle size was studied by sedimentation velocity techniques in the analytical ultracentrifuge. These studies also permitted a comparison of the average particle size in each fraction with measurements made by other techniques. While these studies were not extensive, the data derived provided significant basic information.

The patterns resulting from the ultracentrifugal analyses of chloroplast fragments are shown in Fig. 11. The sedimentation coefficients measured from these patterns are presented in Table 12. CF_{20-50} and CF_{70-145} revealed single broad peaks, indicating that each fraction contained a continuum of particle sizes in a fairly broad size range, yet sufficiently monodisperse to sediment together. This interpretation is borne out by the particle size distributions in the fractions determined from electron microscopy (table 8). The sedimentation constants, ($S_{20,w}$) indicate that the particles in CF_{20-50} are about 6 times larger than those in CF_{70-145}. In each of these fractions a green chlorophyll band was observed to move with the peak indicating that the chlorophyll is associated with the moving boundary.

Table 12

SEDIMENTATION CONSTANTS OF CHLOROPLAST FRACTIONS

Fraction	Peak	Sedimentation Constant, sec x 10^{-13}
CF_{20-50}		800
CF_{70-145}		130
CF_{145s}	a	8
	b	16
	c	36

CF_{145s} shows a multicomponent pattern of at least three moving boundaries (fig. 11). The chlorophyll moved with the minor leading peak, whose $S_{20,w}$ was calculated to be 36. The major component had a 16S sedimentation constant. No green chlorophyll band was associated with this peak or with the small slower moving, 8S peak.

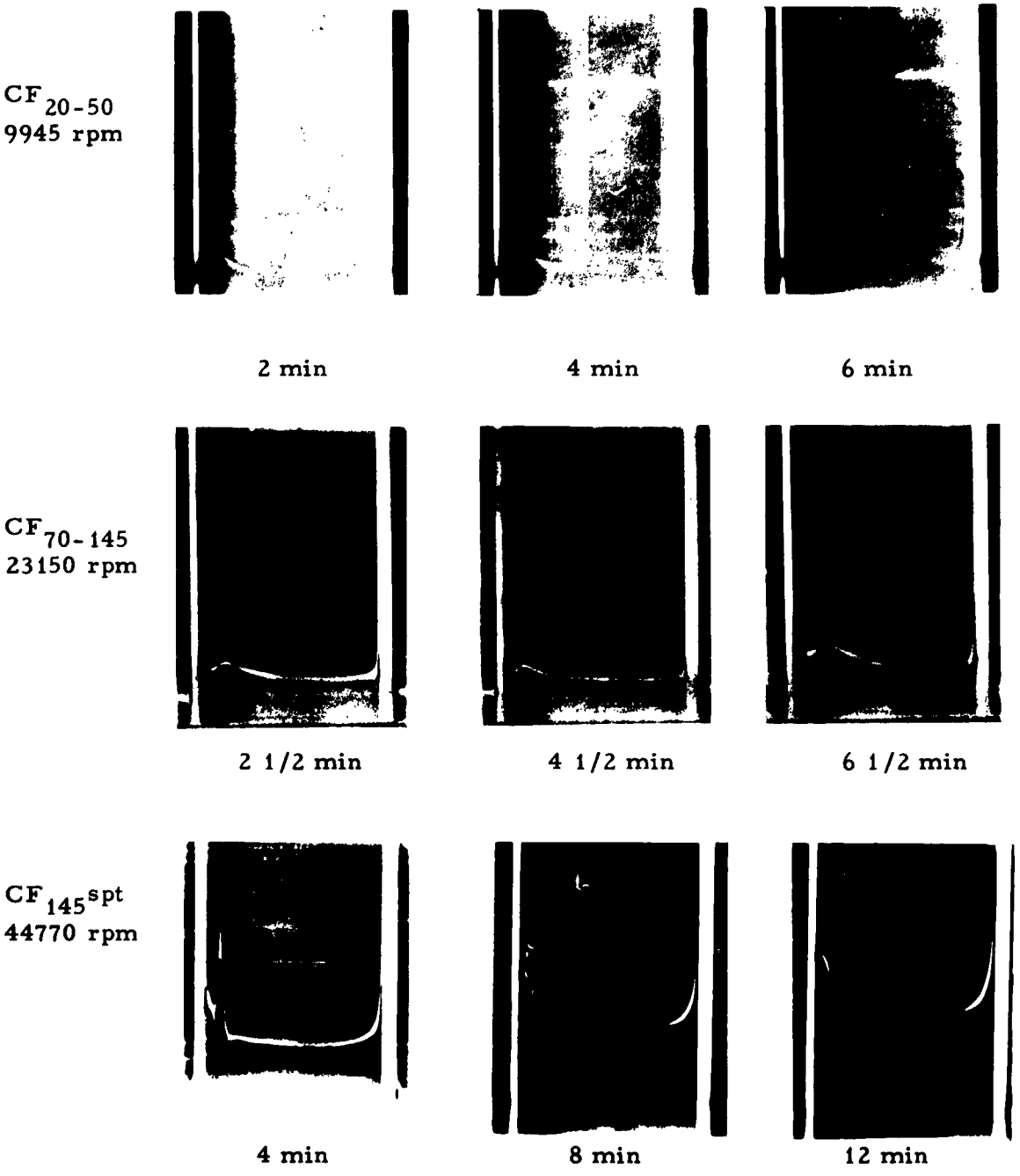


Figure 11

SEDIMENTATION ANALYSIS OF CHLOROPLAST FRAGMENTS

The 16S component is probably equivalent to the 16-18S Fraction I protein of Eggman et al. (ref. 11), Lyttleton and Ts'o (ref. 27), and Park and Pon (ref. 28). These workers analyzed this fraction in buffers of higher ionic strength. The trailing, minor peak is typical of the Fraction I protein pattern, while the 36S peak is not. Although leading peaks have been observed previously, that of Eggman et al. was reported to have appeared only after purification of the fraction by high-speed centrifugation, while that of Lyttleton and Ts'o was eliminated by dialysis or high-speed centrifugation. None of the minor, fast moving peaks was reported to contain chlorophyll. The 36S peak was not removed by dialysis.

A fraction similar to 36S was obtained in the Beckman model L centrifuge using the SW 39 L rotor. CF_{145s} was sedimented at 173,000 x g for 30 min. The sediment was discarded and the supernatant spun at 173,000 x g for 100 min. The green sediment obtained was photoactive, showing a Hill reaction rate of about 40.

It is believed that the particles which sediment at 173,000 x g, probably representing the 36S component in CF_{145s} (fig. 11), are the smallest units known to contain chlorophyll and exhibit Hill activity. Although Thomas et al. (ref. 34) have reported Hill activity from particles smaller than the 36S units and even smaller than the 16S component, their work suffers in that the starting material was not isolated chloroplasts but whole spinach leaves. Consequently, the particle sizes they measured by electron microscopy might not have represented the chlorophyll-containing particles whose activity was measured but rather an inhomogeneous mixture of a few active chloroplast fragments with a large number of nonphotoactive smaller fragments from other cytoplasmic structures.

V. CONCLUSIONS

A. Chemistry-Function

Generally, photosynthetic function is stated in terms of activity per unit of chlorophyll. Chlorophyll is the recognized photosynthetic pigment serving in light-trapping, converting and charge transfer reactions. Yet, as this report indicates, the rate of photosynthetic reaction is not solely dependent on chlorophyll. In the natural state, chlorophyll is linked with protein. Protein too can be used as an index for the Hill reaction. Although there is a parallel relationship between activities based on chlorophyll or on protein (table 6) neither component would be active by itself. Photosynthetic activity is also regulated by other components such as plastoquinone (table 4; refs. 3, 5, 9, 25), β-carotene (ref. 12), vitamin K (ref. 4), possibly a cytochrome (refs. 10, 17, 21, 26), and a newly discovered pigment, allagochrome (ref. 19). In addition Tables 3 and 4 emphasize that the efficiency of the reaction depends upon the balance which exists among the chemical components of the system.

It is not essential that all components be active members of the macromolecular complex in order that they may modify its activity. The effects of the supernatant on the fractions suggest that some lipids and proteins might be present as inert or structural members whose function could be to spatially orient the active molecules. Structural protein has been identified in mitochondria and is postulated to be a general component of cytoplasmic membranes (ref. 16). It seems quite likely that a type of structural protein will also be found to be not only associated with the chloroplast membrane, but also with the organized lamellae and in the chloroplast fragments which display photosynthetic function.

The present experimental data has led to the postulate that a basic photoactive unit exists having a certain minimal composition and organization of chemical components which are absolutely essential for engaging in a photoactive function such as the Hill reaction. The reaction rate in such a particle may be minimal. For maximal efficiency, there must be a cooperative relationship of a number of these basic units into an organized, coordinated larger unit. The number of cooperating subunits necessary for maximal efficiency of the different photosynthetic processes, such as photophosphorylation, oxygen evolution, and carbon dioxide fixation need not be the same. Protein and lipid molecules may be present which are not directly active in the photoprocesses and can be thought of as coordinators or organizers of the subunits, thus modifying their chemical composition and physiological activity.

The fragments comprising CF_{20-50} seem to represent the most efficient organization of subunits for Hill activity. As demonstrated throughout these studies, the activity of CF_{20-50} is the highest of any isolated fraction and can be correlated with a particular quantitative relationship between PQ, chlorophyll, protein and lipid (tables 2 and 4). These components are not the only ones which influence activity, but at present they are the only ones which have been studied in this laboratory. It must be emphasized that it is not just the amounts and ratios of the constituents but also their spatial relationships in the structure which must be responsible for metabolic efficiency. A summary of the chemical-functional interactions, using CF_{20-50} as a base line, is shown in Figure 12.

B. Structure-Function

Previous studies estimated the average particle in CF_{20-50} to be 1500 A in diameter and to contain 1.6×10^4 chlorophyll molecules (ref. 18). The weight of this particle could be assumed as 8×10^7 grams per mole based on its construction from 80 subunits each of 1×10^6 molecular weight. Each subunit was thought of as a 200 x 100 A oblate spheroid, according to Park and Pon (ref. 28). The calculations suggested that a small basic unit existed which had to be oriented into a larger structure to attain maximal functional efficiency. New evidence from electron microscopy and analytical ultracentrifugation confirms the size and approximate molecular weight of the average fragment in CF_{20-50} (fig. 11, table 8, table 12). However, the size and the number of small subunits comprising the large particle remain in question.

It was predicted from sedimentation times that the size range in CF_{20-50} varies from 1500-A-diameter particles, which are the smallest particles which theoretically would be completely sedimented, to particles no greater than 2300 A in diameter. All particles larger than 2300 A would have sedimented during the previous centrifugation at 20,000 x g (CF_{1-20}) (ref. 18). Yet the electron micrographs (fig. 7, table 8) indicate a much wider range of particle sizes, 500 to 6750 A, with some of the 6000 A particles clustered to form 20,000-A units. Since it is unlikely for particles much smaller than 1000 A and virtually impossible for particles larger than 2300 A to have been originally sedimented in CF_{20-50}, the abundant appearance of particles outside the predicted range indicates that a change occurred subsequent to or at the time of centrifugation. Centrifugation procedures could be suspected in these studies, since sediments were washed and centrifuged a second time as a purification step prior to resuspending the fractions for chemical, structural, or physiological assays.

Since, as was shown by electrophoresis (fig. 10), CF_{20-50} is composed of at least three components which may have chemical differences reflected by their surface charge differences, both aggregation and dispersion could result on standing. Alternatively, the increased range of particle sizes noted by electron microscopy may only be an artifact of the method of preparing the specimens for analytical observation. The same general observation and conclusions derived from CF_{20-50} can be drawn for CF_{70-145} (fig. 8). However, the interpretations of electron micrographs are more subjective than are other physical measurements, and these conclusions are drawn with reservations.

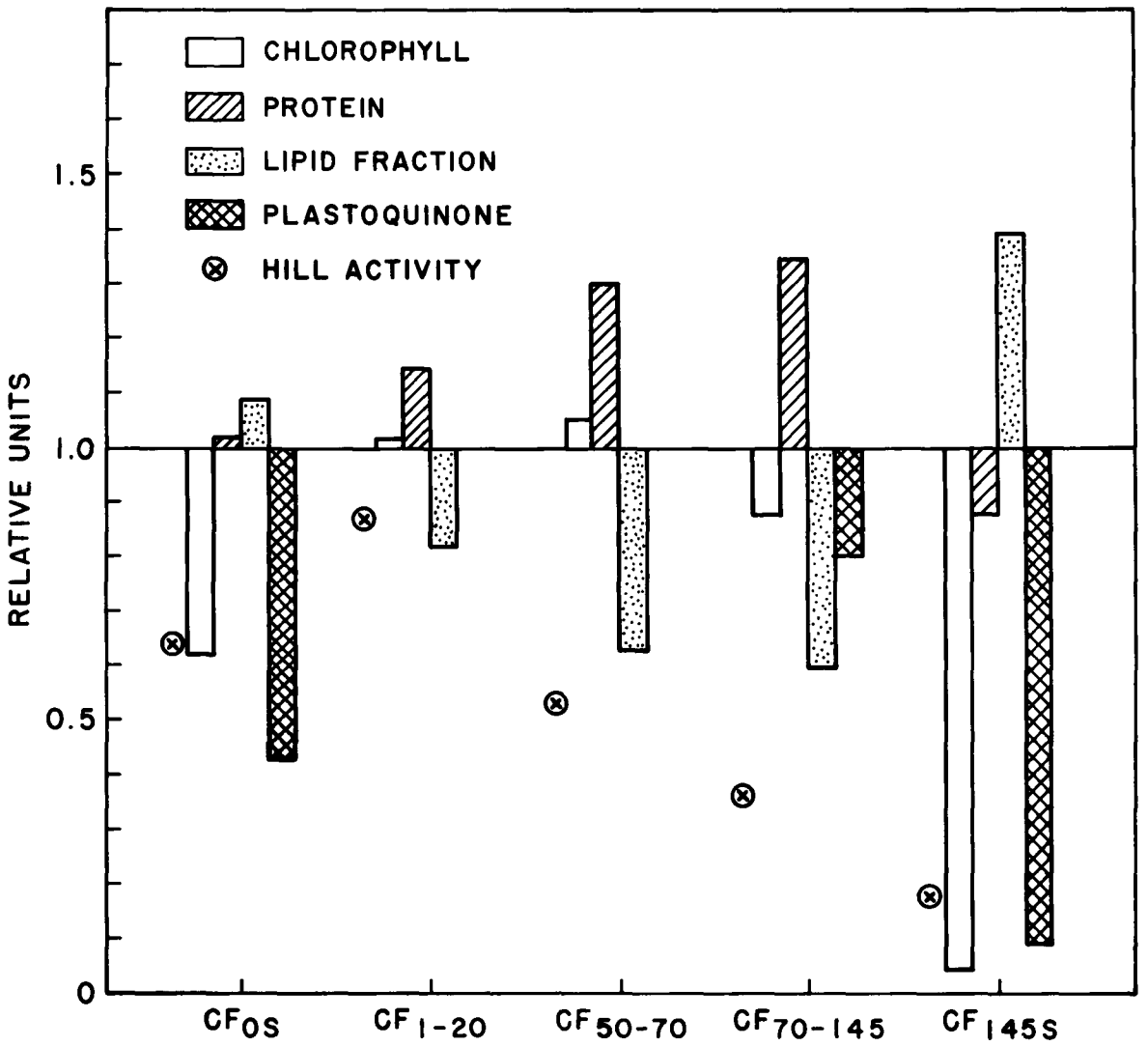


Figure 12

SUMMARY OF THE RELATIONSHIPS BETWEEN HILL ACTIVITY AND CHEMICAL COMPOSITION OF CHLOROPLAST FRACTIONS (CF) NOTE: 1.0 REPRESENTS THE LEVEL OF FACTORS IN CF_{20-50}

CF_{70-145}, a fraction with a Hill activity about one third to one half that of CF_{20-50}, has particles in the relatively narrow size range of 470 to 1000 A in diameter (ref. 18), which averages about one third to one half the size of the CF_{20-50} unit. Sedimentation analysis has shown that the fraction contains a single size-component with a sedimentation constant of 130S (fig. 11, table 12). An approximate molecular weight of 1×10^7 can be calculated for the 130S component on the basis of the geometry of an average particle (fig. 8) and its presumed chemical constitution. The average CF_{70-145} particle, then, could be composed of 7 to 8 of the 200-A oblate spheroids of Park (refs. 28, 29) and could accommodate about 1400 chlorophyll molecules. It would be comparable in size and number of subunits to the quantasome aggregates of Sauer and Calvin (ref. 32).

The data and calculations suggest that particle diameter and activity are related, since CF_{70-145} particles have about one third the diameter as well as one third the activity of CF_{20-50} particles. This apparent correlation between diameter and activity is probably fortuitous. Activity is not correlated with weights, which differ by a factor of 6, or with number of chlorophyll molecules, which differ by a factor of about 12. The activity is related in a complex manner to chemistry; its relationship to structure also appears to be complex. The number, and therefore the structural organization of small units in CF_{70-145} is different from that in CF_{20-50}. These units probably are not spatially arranged in as effective a manner in CF_{70-145} particles as they are in CF_{20-50} fragments.

CF_{145s}, which has shown a Hill reaction rate about one fifth that of CF_{20-50}, is a multicomponent system (fig. 11). It contains the soluble protein of the chloroplast stroma and some chlorophyll-associated protein. The soluble protein is probably Fraction I-type protein, which comprises 70 to 85% of the total amount present (fig. 11) and is thought to be chlorophyll-free. In addition, a minor, heavier component (36S) is present and seems to be chlorophyll-linked. The majority of this component, which was isolated by differential centrifugation at 173,000 x g, exhibits a Hill reaction rate about one fourth that of CF_{20-50}. The estimated size of the largest particles in CF_{145s} was 470 A (ref. 18), which corresponds to this 36S chlorophyll-containing component (fig. 11). The majority of material in the fraction, however, has a sedimentation constant of 16S, which corresponds to the 16S particles of 200-A-diameter (ref. 28). The 36S particles may be two to three times the size of the 16S units if one considers the former to be a lipid-protein complex and therefore less dense than the latter particles which are presumably pure protein. The 36S particles, then could be composed of 2 or 3 of the 200-A particles and be layered with about 700 chlorophyll molecules. The molecular weight of 36S particles would be 2 to 3×10^6 based on the assumed chemical composition.

The admixture in CF_{145s} of a large amount of Fraction I protein, which is not functional in the Hill reaction, with a small amount of the active 36S component could account for the finding of lower activity in the total CF_{145s} than in the chlorophyll-bearing units sedimented from it, because of a supernatant-particulate interaction. The activity difference between these small particles and those from CF_{20-50} are explainable by the rationale used to interpret the difference between CF_{70-145} and CF_{20-50}.

C. Theoretical Considerations

Whether the 36S units represent aggregates of smaller units or are themselves the basic units of photosynthesis remains to be determined. Sauer and Calvin (ref. 32) reported that they have measured the orientation of chlorophyll in quantasomes by electric dichroism. The measurements, however, were made on "quantasome aggregates" of 3 to 8 smaller units according to their reported electron microscope observations. Thus, indirectly their work seems to support the evidence for a large active unit.

The hypothetical quantasomes have apparently never been observed or studied as individually dispersed units. If they are individual units of 200 x 100 A, though different from Fraction I protein, they must have a great affinity for one another and readily form aggregates. This interpretation would suggest that the basic structural unit, the quantasome, is different from the basic functional unit which requires the cooperation of at least two (perhaps three) quantasomes and is therefore comparable to the 36S component. To procede a step further, it can be calculated that 60 to 80 basic structural units or 30 to 40 basic functional units must be present for optimal Hill activity as found in CF_{20-50}. A correlation of the chemical, structural, and physiological findings presented in this report substantiate the concept that not only a certain number but also a particular structural organization of basic units are essential to efficient function.

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The average particle size in CF_{20-50} was 1500 A diameter. The average sedimentation constant was 800; the molecular weight was about 8×10^7; and it showed three electrophoretic bands.

CF_{20-50} showed maximal Hill activity, but was not the smallest photoactive unit studied. Although the nature of the smallest functional unit is uncertain, it is clear that a CF_{20-50} particle is composed of smaller sub-units probably oriented to impart high activity to the larger composite structure.

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